SYNTHESIS OF DNA COMPLEMENTARY TO AMV RNA USING E. COLI POLYMERASE I *

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SUMMARY. E. coli DNA polymerase I has been used to copy avian myeloblastosis virus RNA both in the presence and absence of the primer oligo-dT_{10}. Heteropolymer synthesis is obtained in both cases. The product DNA obtained using the primer has been shown to be complementary to the RNA template. Substantial homopolymer synthesis (poly dT and poly (dA)·(dT)) occurs in the presence and very little in the absence of the oligo dT_{10} primer.

We have recently shown that a complementary DNA product is obtained using E. coli polymerase I with rabbit globin mRNA as a template (1). Those results, together with the fact that various ribosomal RNAs can serve as templates with this enzyme (2,3,4), indicated that oncornaviral RNAs might also be able to act as templates for the synthesis of complementary DNA with E. coli polymerase I. The results presented in this report bear out this expectation. In addition to its theoretical interest, the process of reverse transcription by the readily available E. coli enzyme makes it possible to produce DNA probes from various RNAs with little difficulty.

Materials: DNA polymerase I (fraction 7) was kindly supplied by Dr. L. Loeb. Its specific activity was about 230,000 nmoles/mg using activated DNA in a 20 minute assay. AMV plasma was generously made available through Dr. M.A. Chirigos of the National Institutes of Health and supplied by Dr. J. Beard. RLV (tissue culture grown) was supplied by Dr. J. Gruber. Aspergillus S-1 nuclease was a generous gift of Dr. Akhil Vaidya. Viral 70S RNA was prepared using the standard procedure of Kacian et al (5) which consists of disrupting purified virus in 0.5% SDS in Tris-NaCl-EDTA (TNE) (0.01M Tris, pH 7.8-0.15M NaCl-0.001M EDTA) buffer, followed by extraction with phenol-cresol, precipitation of the nucleic acid by ethanol, and final purification on a glycerol gradient. 70S fractions were pooled, reprecipitated and stored in small aliquots. Other methods are described in the legends to the figures and the tables.

RESULTS:

70S AMV RNA as Template (no added primer): Synthesis of heteropolymeric

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DNA on 70S AMV RNA in the absence of any added primer can be demonstrated by labelling with any one of the four substrate triphosphates in the presence of either Mg^{++} or Mn^{++} (Table 1). The reaction is dependent on the presence of

TABLE 1

Homopolymeric and Heteropolymeric Synthesis with AMV 70S RNA

Substrate	picomoles incorporation/μg RNA			
	RNA alone		RNA + oligo dT_{10}	
	Mg^{++}	Mn^{++}	Mg^{++}	Mn^{++}
^{3}H-TTP alone	0.5	> 0.05	60.0	45.0
" + dATP	0.6	0.6	270.0	570.0
" + dATP + dCTP + dGTP	2.4	2.4	135.0	225.0
" + " " "				
+ oligo dT_{10}, minus RNA	< 0.05	< 0.05	< 0.05	< 0.05
^{3}H-dATP alone	0.3	< 0.05	< 0.1	< 0.1
" + TTP	0.42	< 0.05	18.0	25.0
" + TTP + dCTP + dGTP	1.5	1.1	30.0	15.0
+ " " "				
+ oligo dT_{10}, minus RNA	< 0.05	< 0.05	< 0.05	< 0.05
^{3}H-dGTP + TTP + dCTP + dATP	1.4	2.0	1.7	4.0
^{3}H-dCTP + TTP + dGTP + dATP	1.6	2.2	1.8	4.4

Assays were carried out in final volume of 0.1 ml containing: 50mM Tris:HCl (pH 7.8), 5mM mercaptoethanol, 4 μM of each of indicated triphosphates, 50 μg/ml albumin, 5mM $MgCl_2$ (or 0.5mM $MnCl_2$ and 0.05M KCl in the Mn^{++} system). The specific activity of the labelled substrate was 750 cpm/pmole. 0.2 μg of AMV 70S RNA either alone or hybridized with 0.001 μg of oligo dT_{10} (and 0.04 μg of fraction 7 of Kornberg polymerase) were used in each assay. All incubations were for 30 minutes at 37°C. Reactions were stopped by adding TCA to 5%. Precipitates were collected on GF/B Whatman filters and counted in a Beckman LS-350 counter.

all four deoxynucleoside triphosphates. There is also some homopolymeric synthesis as shown by the incorporation when the single substrates ^{3}H-TTP or ^{3}H-dATP are used (Table 1). In the presence of all four deoxynucleoside triphosphates and Mg^{++}, heteropolymer synthesis is about 5-fold greater than

homopolymer synthesis. In the Mn^{++} system, the ratio of heteropolymer to homopolymer synthesis is even greater.

70S AMV RNA as template with oligo dT_{10} as primer: Using this primer-template, three types of polymer synthesis can occur (Table 1). The situation is identical to the one which we observed using globin mRNA annealed to oligo dT_{10} as primer-template (1) except that poly (dA)·(dT) production is much greater in the 70S RNA-oligo dT_{10} reaction. It is clear from Table 1 that TTP alone can be incorporated into a poly dT product, initiated on oligo dT_{10} annealed to a poly A stretch presumably situated at the 3' end of the 70S RNA molecule. Incorporation of dATP is dependent upon the addition of cold TTP to the reaction mixture. It is, therefore, impossible to determine the extent of true heteropolymeric synthesis using labeled TTP or dATP in the presence of all four triphosphates, because of concommittant homopolymeric and copolymeric synthesis. The synthesis of heteropolymer has been followed with labelled dGTP or dCTP. From the data shown in Table 2, it is clear that incorporation of dGTP label into polymer can occur only if all of the four deoxynucleoside triphosphates are present. Furthermore, the heteropolymeric reaction is relatively insensitive to actinomycin D, and sensitive to RNAse, implying that the synthesis is truly RNA-dependent. The overall homopolymeric or heteropolymeric synthesis is much greater in the presence of Mn^{++} than in the presence of Mg^{++}. For the work, described in Figs. 1-4 and Table 3, we have employed RNA annealed

TABLE 2

Heteropolymeric Synthesis with AMV 70S RNA

	picomoles incorporation/μgRNA			
	RNA alone		RNA + Oligo dT_{10}	
	Mg^{++}	Mn^{++}	Mg^{++}	Mn^{++}
^{3}H-dGTP + dATP + dCTP + TTP	1.4	2.0	1.7	4.0
" minus dATP	0.2	0.25	0.2	0.25
" minus dCTP	0.3	0.4	0.4	0.4
" minus 3 dXTPs	0.2	0.01	0.3	0.01
" minus RNA, minus RNA-Oligo dT_{10}	0.1	0.0	0.0	0.0
" plus RNAse pretreatment (100 μg/ml-5' at 37°C)	-	< 0.2	-	< 0.2
" plus Actinomycin D (100 μg/ml)	-	1.8	-	3.4

Reaction conditions were identical to those described in Table 1. Mn^{++} system contained 0.5mM $MnCl_2$ and 0.05M KCl and Mg^{++} system had 5mM $MgCl_2$.

TABLE 3

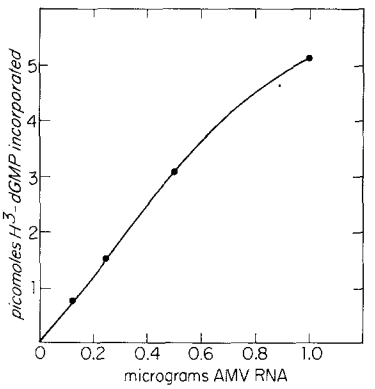
Back-Hybridization of the DNA Product to AMV 70S RNA

Sample	% Resistant to S_1 Nuclease
1. Product ^{3}H-DNA alone (without RNA)	10
2. Product ^{3}H-DNA + mRNA annealed for 60 minutes	65
3. Same as 2 above except for 120 minutes	70
4. Product ^{3}H-DNA annealed for 120 minutes with rat liver 28S ribosomal RNA	10
5. Same as 4 except with EMC RNA	10
6. " " " " " RLV RNA	10

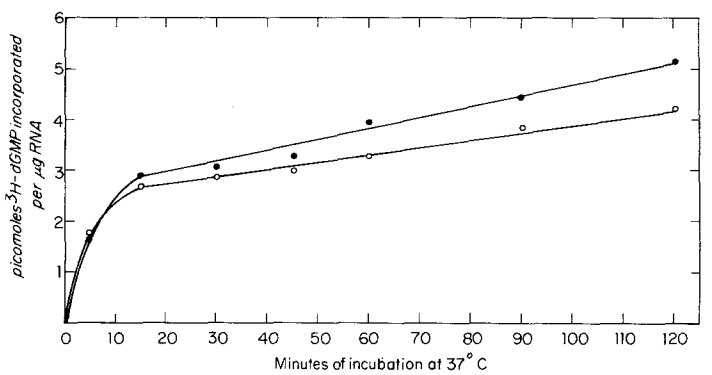
Synthesis of ^{3}H-DNA was carried out as described in Table 2 except that the reaction volume was ten-fold higher. The reaction mixture was extracted with standard phenol-cresol 8-hydroxyquinoline mixture; 100 µg of denatured calf thymus DNA was added to the aqueous layer and was precipitated with 2 volumes of ethanol. The precipitate was collected by centrifugation and dissolved in 40 µl of 0.5N NaOH. The solution was drawn up in a capillary, sealed and incubated overnight at 37°C. The contents were neutralized with concentrated HCl; the final volume was about 50 µl. 5 µl aliquots were used for each assay. A control (100%) was put through the procedure without mRNA and with no subsequent nuclease treatment. Sample 1 was the same except that it was treated with the nuclease. The hybridization was carried out as follows. To 5 µl aliquots was added 5 µl of 6XSSC containing 2 µg AMV-70S RNA, 2 µg of RNA for samples 4 and 5 and 8 µg of RLV RNA for sample 6. After thorough mixing on a sheet of parafilm, the mixture was drawn into a 50 µl capillary tube and sealed at both ends. The tubes were then immersed in a waterbath at 69°C for the desired time, and then chilled in ice. The contents of each tube were then poured into a tube containing 0.2ml of S_1 nuclease assay mixture (consisting of 0.02M Na-Acetate buffer, pH 4.5, 0.3M NaCl, 0.003M $ZnCl_2$, 10 µg albumin/ml and heat denatured calf thymus DNA at 10 µg/ml). The reaction was incubated for 2 hrs at 50°C after addition of 1 unit of S_1 Aspergillus nuclease, and acid-insoluble counts were determined by TCA precipitation.

to oligo dT_{10} as primer-template and the $MnCl_2$-KCl assay system.

Kinetics of the reaction and the effect of Actinomycin D: Figure 1 shows the synthesis of heteropolymeric product with increasing amount of 70S RNA-oligo dT_{10}. The extent of synthesis is directly proportional to the amount of RNA added as template, indicating that the quantity of enzyme used (0.04 µg) is not rate-limiting up to 1 µg of template RNA. Figure 2 shows the kinetics of incorporation in the presence and absence of Actinomycin D, which has little effect, as expected, when using an RNA template (6). Most of the synthesis is completed in the first 15 minutes and continues at a greatly decreased rate



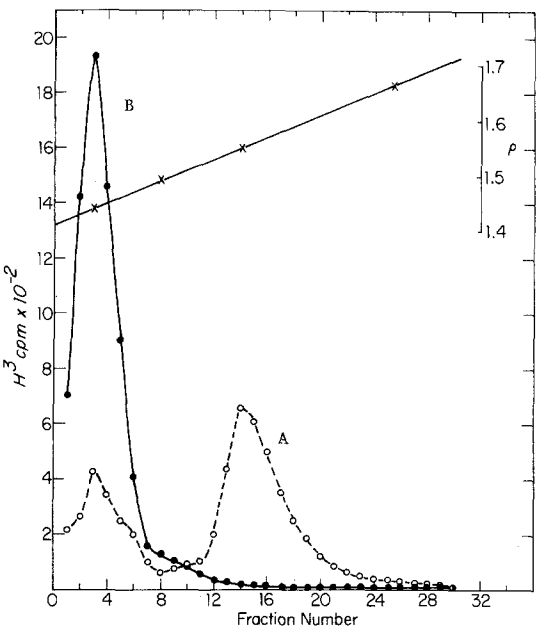
Legend to Fig. 1: DNA synthesis as a function of AMV RNA-oligo dT_{10} concentration. The reaction mixture is given in the legend to Table 1, using the $MnCl_2$-KCl system. The amount of AMV RNA:oligo dT_{10} was varied as indicated. Samples were processed as in the legend to Table 1.



Legend to Fig. 2: Kinetics of incorporation using AMV-oligo dT_{10} as primer-template in the presence (—O—O—) and absence (—●—●—) of Actinomycin D (50 µg/ml). 50 µl portions were withdrawn at various times from a 500 µl reaction mixture described in Table 1. Samples were processed as in Table 1.

thereafter. The reaction is insensitive to Actinomycin D to the extent of about 90% during the initial period of synthesis.

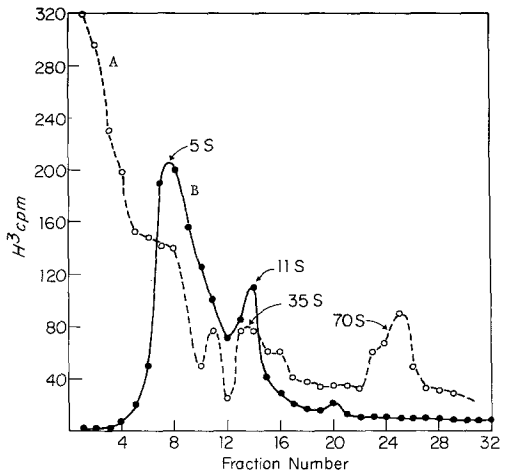
Products of the reaction: Figure 3 shows the size of the heteropolymeric DNA product formed using AMV 70S RNA-oligo dT_{10} as the primer-template. A 10-30% glycerol gradient was used for the sedimentation of the native product, which exhibited S values of 70, 35 and smaller (curve A). Presumably, the slower sedimenting species arise from synthesis on RNA moieties that have



Legend to Fig. 3: Sedimentation velocity gradients of native product (curve A) and alkali-treated product (curve B). Heteropolymeric product using ^{3}H-dGTP as the label was synthesized on a large (1.0 ml) scale using the Mn^{++}-KCl system described in Table 1. Incubation was for 15 minutes in the presence of Actinomycin D (50 µg/ml). The reaction was stopped by the addition of EDTA to 10mM and extracted with an equal volume of phenol-cresol mixture. One half of the aqueous phase was placed on 13 ml of a 10-30% glycerol gradient made with TNE buffer. Centrifugation was for 3 1/2 hours at 4° and 40,000 rpm in an SW 40 rotor. AMV RNA was used as a marker in a companion tube. The other half of the solution was made 0.5M in NaOH, heated to 100° for 30 minutes and neutralized. It was placed on a 13 ml 5-20% sucrose gradient and run as before, except for 14 hrs. The markers were 5S and 16S ribosomal RNAs and were run in parallel gradients. Marker positions are indicated by arrows. Fractions were collected from the top, precipitated with TCA and counted in a Beckman LS 350 counter.

arisen from degradation of the 70S RNA. Alkaline treatment of this product shows a much smaller distribution of sizes in a 5-20% sucrose gradient of the single stranded DNA, Fig. 3 (curve B). Two discrete sizes are apparent, one sedimenting at 5S; the other at 11S.

Figure 4 gives the results of cesium sulfate density gradient centrifugation of the heteropolymeric product before (curve A) and after (curve B) treatment with alkali. Before alkali, there is a peak at density 1.44 which corresponds to single-stranded DNA, as well as material in the denser region (ρ = 1.55-1.56). The latter indicates that a significant amount of newly-synthesized DNA is associated with template RNA; alkali treatment, as expected, completely eliminates the hybrid material, which is then recovered in the lighter region (ρ = 1.44). A significant amount of the back-hybridized prod-



Legend to Fig. 4: Cesium sulfate density gradient centrifugation of AMV RNA-oligo dT_{10} product. Native (curve A) and alkali treated (curve B) products were made as described in the legend to Fig. 3. 0.2 ml of the aqueous phase were applied on top of a Cs_2SO_4 step gradient ranging in 4 steps from ρ = 1.41 to 1.66. The run was at 40,000 rpm for 62 hrs at 22° in a 50 Ti rotor. Aliquots were collected from the top, precipitated with TCA and counted in the LS 350 counter.

uct (Table 3) appears in the region of between 1.49-1.58 (data not shown).

Hybridization of DNA product to AMV 70S RNA: In order to show that the heteropolymeric DNA product is complementary to AMV RNA, back-hybridization experiments were performed. The resistance of the annealed hybrid to degradation by the Aspergillus single-strand-specific (S_1) nuclease was used as the basis for determining hybrid formation. The DNA product of the reaction, as described in Table 3, was freed of native RNA by alkali treatment. After neutralization, it was annealed with 2 μg of 70S AMV RNA for 60 and 120 min. Approximately 70% of the input counts were resistant to treatment with S_1 nuclease (Table 3). The specificity of hybridization is shown by the fact that neither 28S ribosomal, EMC nor RLV RNAs hybridized to the DNA product. The 10% resistance to S_1 nuclease in these cases is probably due to self-annealing of the DNA product, as shown by the control which lacks RNA (Table 3)

DISCUSSION: We have demonstrated that E. coli DNA polymerase I can utilize AMV RNA as template to produce a complementary DNA product in the presence of either Mg^{++} or Mn^{++}. An oligo dT_{10} primer enhances the template activity of the RNA to produce a heteropolymeric product. Back hybridization shows that the product is complementary to AMV RNA. Much of the product bands with the RNA in a Cs_2SO_4 gradient, and also sediments with the RNA on sucrose gradients.

The heteropolymeric DNA product synthesized in the presence of oligo dT_{10} consists of two species, as shown by sedimentation analysis after alkali treat-

ment. We suspect that these two species are synthesized at different initiation sites on the template. One of these is presumed to be the poly A terminus, to which is annealed the oligo dT_{10} primer; the other could be either an internal poly A sequence, also annealed to oligo dT_{10}, or, since synthesis can also take place in the absence of oligo dT_{10}, an initiator site(s) occurring naturally in the RNA. Different initiation sites could give rise to different molecular weight species of DNA product depending on their location.

The size of the DNA product (5S and 11S) is significantly smaller than the template. However, the same is true of viral reverse transcriptase products where the average size is about 5S (8,9). It appears that the secondary structure of 70S RNA may be a limiting factor permitting only small single-stranded regions to be copied.

The density of the main heteropolymeric product formed in the presence of oligo dT_{10} primer is about 1.55, which is lower than that (1.68) reported using viral reverse transcriptase in the absence of the primer oligo dT_{10}. Presumably, the ratio of DNA to RNA is higher in our product. Thus, it appears that in the presence of oligo dT_{10}, a greater proportion of the template is copied, as would be expected if one or more additional initiation sites are provided. A detailed investigation of these products synthesized with E. coli polymerase I is being carried out, including a comparison with those obtained with viral reverse transcriptase under identical conditions.

Analogous to our earlier studies (1) of the products obtained using globin mRNA as template, we find that poly dT and poly (dA)·(dT) are synthesized (in amounts several times greater than heteropolymeric DNA) when oligo dT_{10} is added to the AMV RNA as primer. Consequently, under these conditions heteropolymer synthesis can be monitored with radioactivity in dCTP or dGTP but not in dATP or TTP. In the presence of oligo dT_{10} primer, synthesis is more extensive with Mn^{++} than with Mg^{++}.

Preliminary results obtained using RLV RNA as a template have yielded results similar to those described for AMV RNA. We plan to carry out a critical analysis of the regions copied by E. coli polymerase I of these RNAs and to compare them with those obtained using the reverse transcriptase. We also plan to examine the relative efficacy of the DNA products made by these two enzymes as probes in detecting the viral-related sequences found in normal or malignant tissues.

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